Bioactivation of N-nitrosomethylbenzylamine and N-nitrosomethyl-amylamine in oesophageal papillomas

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Abstract: Oesophageal papillomas were induced in male F344 rats by continuous exposure to N-nitrosomethylbeazylamine (NMBZA) and N-nitrosomethyl(2-methylbutyl)ainine in the drinking water at concentrations of 10 and 19.5 p.p.m. respectively. After 81-141 days animals received a single i.p. chasing dose of NMIBZA (0.1 mmol/kg), [14C-methyl]NMBZA or N-nitroso[14C-methyl]amylamine and were killed 6 h later. Induced papillomas (3-9 per animal) were analysed by autoradlography and by immunohistochemistry using a polyclonal antibody to O6-methyldeoxyguanosine Both techniques revealed the presence of high levels of alkylation products in all papillomas investigated. Immunohistochemical staining of O6-methyldeoxyguanosine was largely restricted to nudei of the basal layer and of epithelial cells with incipient keratinization. These findings demonstrate that NMBZA and N-nitrosomethylamylamine and probably related methyl alkylnitrosamines are effectively bioactivated in premalignant lesions, indicating that during chronic exposure papifiomas can acquire additional mutations that are likely to play a major role in tumour progression

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Bioactivation of *N*-nitrosomethylbenzylamine and *N*-nitrosomethylamylamine in oesophageal papillomas

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Oesophageal papillomas were induced in male F344 rats by continuous exposure to *N*-nitrosomethylbenzylamine (NMBzA) and *N*-nitrosomethyl(2-methylbutyl)amine in the drinking water at concentrations of 10 and 19.5 p.p.m. respectively. After 81–141 days animals received a single i.p. chasing dose of NMBzA (0.1 mmol/kg), [^{14}C-methyl]NMBzA or *N*-nitroso[^{14}C-methyl]amylamine and were killed 6 h later. Induced papillomas (3–9 per animal) were analysed by autoradiography and by immunohistochemistry using a polyclonal antibody to O^6-methyldeoxyguanosine. Both techniques revealed the presence of high levels of alkylation products in all papillomas investigated. Immunohistochemical staining of O^6-methyldeoxyguanosine was largely restricted to nuclei of the basal layer and of epithelial cells with incipient keratinization. These findings demonstrate that NMBzA and *N*-nitrosomethylamylamine and probably related methylalkylnitrosamines are effectively bioactivated in premalignant lesions, indicating that during chronic exposure papillomas can acquire additional mutations that are likely to play a major role in tumour progression.

Introduction

N-Nitrosomethylbenzylamine (NMBzA*), *N*-nitrosomethylamylamine (NMAA) and related asymmetric *N*-nitrosodialkylamines selectively induce tumours of the rat oesophagus, largely irrespective of the mode of administration (1). There is evidence that this organ specificity is due to the presence of cytochrome P450 isozymes in rat oesophagus which efficiently bioactivate this group of carcinogens by α-C hydroxylation at either the methyl group or at the opposite aliphatic or aromatic moiety (2–4). Biochemical studies have revealed that the latter pathway, which leads to the formation of a methylating intermediate, predominates and that methylated DNA bases are likely to be responsible for tumour initiation by this group of carcinogens (5–7). Immunohistochemical studies using antibodies specific for the promutagenic DNA-base O^6-methyldeoxyguanosine have shown that bioactivation occurs in the mucosa proper, with alkylated nucleic detectable predominantly in the basal cell layer (8–10).

Histological analyses have revealed that NMBzA and related asymmetric methylalkylnitrosamines typically induce papillomas as early benign precursor lesions. Some of these papillomas ultimately progress to form squamous carcinomas. In the present study, we have investigated whether and to what extent papillomas maintain the capacity to bioactive the nitrosamines responsible for their induction. Using autoradiographic and immunohistochemical techniques, it could clearly be shown that all papillomas investigated bioactivate NMBzA and related agents, indicating that during continuous exposure additional mutations can be induced in these premalignant lesions.

*Abbreviations: NMBzA, *N*-nitrosomethylbenzylamine; NMAA, *N*-nitrosomethylamylamine; NM2MBA, *N*-nitrosomethyl(2-methylbutyl)amine; BUdR, 5-bromodeoxyuridine

Materials and methods

Chemicals and antibodies

[^{14}C-methyl]NMBzA was synthesized according to Skipper (11). The radiochemical purity was >95%. [^{14}C-methyl]NMAA was prepared as described previously (9). *N*-nitrosomethyl(2-methylbutyl)amine (NM2MBA) was from the German Cancer Research Centre, Heidelberg, FRG. RNase T_1 from *Aspergillus oryzae* was from Boehringer-Mannheim AG, Rotkreuz, Switzerland. Disulfiram, RNase A from bovine pancreas, ovalbumin and 3,3-diaminobenzidine–4HCl were purchased from Sigma Chemie, Deisenhofen, FRG. Peroxidase-(rabbit)anti-peroxidase complex, swine anti-rabbit Ig (raised against rabbit serum pool), biotinylated rabbit anti-mouse Ig, non-immune swine and rabbit sera and avidin–biotin peroxidase complex were obtained from Dakopatts AS, Glostrup, Denmark. Monoclonal anti-BUdR antibody was from Becton-Dickinson, Basel, Switzerland. Characteristics of the rabbit antiserum raised against keyhole limpet haemocyanin conjugates of O^6-methylguanosine (NPZ 193-1) have been described earlier (12). The antiserum was used without prior absorption. All other chemicals were of analytical grade or higher.

Tumour induction

Male Fischer 344 rats with an average weight of 104 g were obtained from Charles River Wiga, Sulzfeld, FRG. The animals were maintained on water *ad libitum* to which NMBzA or NM2MBA was added as indicated in Table I. Rats exposed to NMBzA were given a semi-synthetic rat chow (no. 2040; Klingentalmühle, Kaiseraugst, Switzerland) to which disulfiram was added at a final concentration of 200 mg/kg: 1 kg of the pulverized pellets and 200 mg disulfiram were mixed with 600 ml of water, formed into pellets (30–50 g) and air dried for 3 days. Disulfiram decreases the metabolism of NMBzA in rat liver, leading to extended exposure to the carcinogen and thus to higher levels of DNA methylation in extrahepatic tissues. This metabolic shift is associated with a marked acceleration of oesophageal tumorigenesis (13,14).

Chasing dose

A single chasing dose of NMBzA [^{14}C-methyl]NMBzA or [^{14}C-methyl]NMAA was administered by i.p. injection as indicated in Table I. After 6 h (NMBzA) or 2 h ([^{14}C-methyl]NMBzA, [^{14}C-methyl]NMAA), the animals were killed by exsanguination during ether anaesthesia.

Autoradiography

For autoradiographic studies, rats were pretreated with an i.p. injection of hydroxyurea (500 mg/kg) followed 30 min later by an i.p. dose of the labelled nitrosamine (Table I). Tissues were fixed in buffer formaldehyde (4%, v/v), embedded in paraffin, and cut into 4 μm sections. Exposure of contact autoradiographs (LKB ^{3}H-Ultrofilm) was carried out for 3 weeks in an X-ray cassette. Films were processed with Kodak D19 developer and Kodak Unifix fixation salt.

Immunohistochemistry for O^6-methyldeoxyguanosine

The organs were removed rapidly and quickly frozen onto small aluminum plates placed directly on slabs of dry ice. Cryostat sections (6–10 μm) were mounted on ovalbumin-coated slides. Immunohistochemical staining was carried out essentially according to Scherer *et al.* (15) as detailed earlier (9). Endogenous peroxidase was inactivated by a 45 min incubation with 0.3% H_2O_2 in methanol. After rehydration via graded ethanol, sections were equilibrated with 10 mM EDTA in 10 mM EDTA in 10 mM Tris, pH 8.0, for 5 min and subsequently treated for 60 min at 37°C with RNase A (200 μg/ml) and RNase T_1 (50 U/ml) in the same buffer. The sections were then rinsed with distilled water, equilibrated in 40% ethanol for 1 min and treated for 10 min at room temperature with 50 mM NaOH in 40% ethanol to denature the DNA, quickly neutralized with 5% glacial acetic acid in 40% ethanol, washed once with water, incubated for 5 min in wash

buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% gelatine, 0.05% Triton X-100), and then washed in PBS (123 mM NaCl, 8.3 mM Na_2HPO_4, 3.2 mM KH_2PO_4, pH 7.4). The sections were subsequently preincubated (60 min, 37°C) with antibody dilution buffer (10% heat-inactivated non-immune swine serum in PBS containing 0.04% Triton X-100). The reaction with the anti-O^6-methyldeoxyguanosine serum (diluted 1:10 000) was carried out for 16 h at 4°C. After this and all of the following incubation steps the sections were rinsed once with PBS, once with wash buffer and again with PBS. Bound antibodies were visualized by the 'double PAP' staining procedure, which involved successive incubations with swine anti-rabbit Ig, peroxidase-(rabbit)antiperoxidase complex, swine anti-rabbit Ig, and peroxidase-(rabbit)antiperoxidase complex, each for 45 min at room temperature. Enzymatic activity was revealed by incubation in 50 mM Tris–HCl, pH 7.4, 3,3-diaminobenzidine–4HCl (0.5 mg/ml) and 0.015% H_2O_2 for 5–10 min at room temperature. The sections were washed with distilled water, lightly counterstained with H&E, dehydrated and mounted.

Bromodeoxyuridine (BUdR) immunohistochemistry

BUdR was given as a single i.p. dose (20 mg/kg body wt). Two hours later, animals were killed and tissues were fixed in 70% (v/v) ethanol overnight, dehydrated and embedded in paraffin. Slides were deparaffinized and stained with a monoclonal antibody to BUdR, followed by incubations with biotinylated rabbit-anti-mouse Ig antibodies, avidin–biotin horseradish peroxidase and 3,3-diaminobenzidine–4HCl (16,17). Sections were counterstained with haematoxylin.

Results and Discussion

The aim of the present study was to determine whether early neoplastic lesions in the rat oesophagus retain the ability to bioactivate asymmetric methylalkylnitrosamines. Two methods of investigation were used. After administration of ^{14}C-labelled carcinogens, autoradiography with dehydrated, paraffin-embedded sections was used to visualize ^{14}C radioactivity bound to cellular macromolecules, including DNA, RNA and proteins. In order to reduce metabolic incorporation of the label via the C1-pool, which may occur to a significant extent in tissues with a high cell turnover, the survival time was kept short (2 h) and animals were pretreated with a single i.p. dose of hydroxyurea (500 mg/kg) which causes almost complete inhibition of DNA synthesis for 3 h (18). We have previously shown that in rat duodenum, i.e. a tissue notorious for high metabolic incorporation, treatment with hydroxyurea reduces purine labelling by >98% (9). The autoradiography shown in Figure 1, prepared after a chasing dose of [^{14}C-methyl]NMBzA, clearly demonstrates that adduct formation is not restricted to the oesophageal mucosa. The papillomas induced by NM2MBA show similarly intensive labelling. Comparison with an adjacent section stained with H&E reveals that only the heavily keratinized portions void of viable nuclei lack ^{14}C labelling. Similar results were obtained on autoradiographs of other papillomas in the same animal and also when [^{14}C-methyl]NMAA was used as the chasing carcinogen (not shown).

Immunohistochemistry offers the advantage of identifying cells containing specific DNA adducts (19,20). In the present study, we used a rabbit antiserum to O^6-methyldeoxyguanosine (12). When this technique was applied to oesophagi of rats chronically exposed to NMBzA or NM2MBA in the drinking water, only very few immunoreactive cells were detected in the basal layer of the adjacent oesophageal mucosa, in regions with dysplasia and in papillomas (not shown). This is in agreement with earlier observations (10) showing that this promutagenic base does not accumulate in the target tissue. It appears that the high cell turnover combined with moderately active DNA repair (21) leads to a rapid loss of adduct from target organ DNA. We have therefore chosen to use a single moderate to high chasing dose in order to determine to what extent oesophageal papillomas, i.e. benign precursor lesions, maintain the capacity to enzymatically convert methylalkylnitrosamines to their ultimate methylating species. We found that during chronic exposure the oesophageal mucosa undergoes thickening and hyperkeratosis and this is paralleled by an increased number of methylated nuclei both in the basal layer and in cells with incipient keratinization (Figure 2A). Similarly, papillomas exhibited a remarkable potential for nitrosamine bioactivation. Again, immunoreactive nuclei were located in the basal layer (Figure 2B) and in mucosal cells migrating towards luminal surface (Figure 2C). Using an antibody to BUdR, it was further established that nitrosamine-induced papillomas exhibit considerable proliferative potential: following a single dose of BUdR, numerous S-phase nuclei were detected throughout the papillomatous mucosa (Figure 2D). Our results with oesophageal papillomas are in agreement with recent observations on the metabolism of the pancreatic carcinogen azaserine by azaserine-induced foci and nodule cells of rat pancreas (J.Bax and E.Scherer, unpublished results). They contrast, however, with the observations in preneoplastic liver foci, which consistently lose the ability to metabolize hepatocarcinogens (8,22), probably due to the increased expression of P-glycoproteins (23,24) and the decreased expression of specific cytochrome P450 isozymes (25).

Demonstration of the fully retained capacity for nitrosamine bioactivation of oesophageal papillomas is of considerable significance for our understanding of the mechanism of malignant transformation in this model system of target organ specificity. Since oesophageal neoplasms in rats are usually induced by chronic exposure, it must be assumed that the formation of promutagenic DNA adducts and of somatic mutations derived thereof continues throughout the early stages of initiation and during clonal expansion of initiated cells to benign papillomas. Recent molecular genetic investigations have shown that NMBzA-

Table I. Treatment of animals used for the demonstration of nitrosamine bioactivation in oesophageal papillomas

Carcinogen	Exposure[a] (p.p.m.)	Duration (days)	Total dose (mg)	Chasing carcinogen	Chasing dose (mg/kg)
NMBzA[b]	10.0	141	19.7	NMBzA[c]	15.0
NMBzA[b]	10.0	81	10.5	NMBzA[c]	15.0
NMBzA[b]	10.0	81	9.1	NMBzA[c]	15.0
NM2MBA	19.5	108	35.4	[^{14}C-methyl]NMBzA[d]	4.9
NM2MBA	19.5	108	35.2	[^{14}C-methyl]NMAA[e]	9.1

[a]Refers to carcinogen concentration in the drinking water.
[b]Co-administered with disulfiram (200 mg/kg food pellets).
[c]Administered by i.p. injection. Chronic exposure to the carcinogen in the drinking water was discontinued immediately thereafter.
[d]Administered by i.p. injection 17 days after cessation of chronic exposure to NM2MBA. Rats received a single s.c. dose of hydroxyurea (500 mg/kg) 30 min prior to the carcinogen.
[e]Administered by i.p. injection 14 days after cessation of chronic exposure of NM2MBA. Rats received a single s.c. dose of hydroxyurea (500 mg/kg) 30 min prior to the carcinogen.

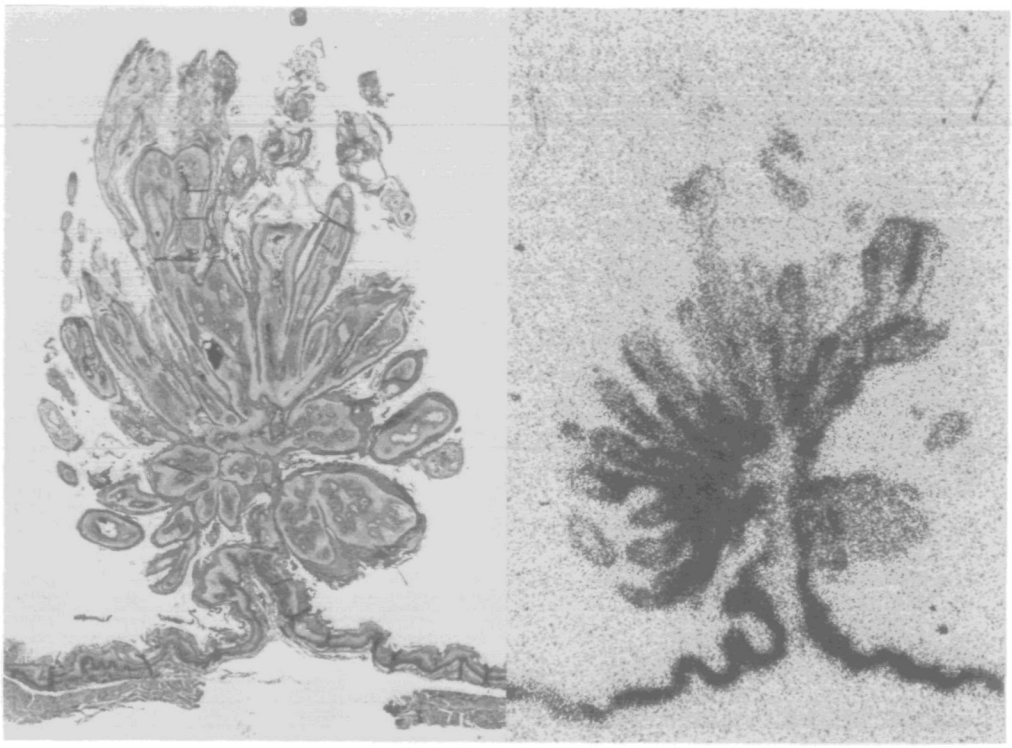

Fig. 1. *Histopathology (H&E stain,* **left***) and autoradiography (***right***) of a papilloma induced in a male F344 rat by chronic exposure to NM2MBA (19.5 p.p.m. in the drinking water), following a chasing i.p. dose of [^{14}C-methyl]NMAA (9.1 mg/kg body wt; survival time 2 h). In order to suppress DNA synthesis, the animal was given a single i.p. injection of hydroxyurea 30 min prior to nitrosamine application. ×17.7.*

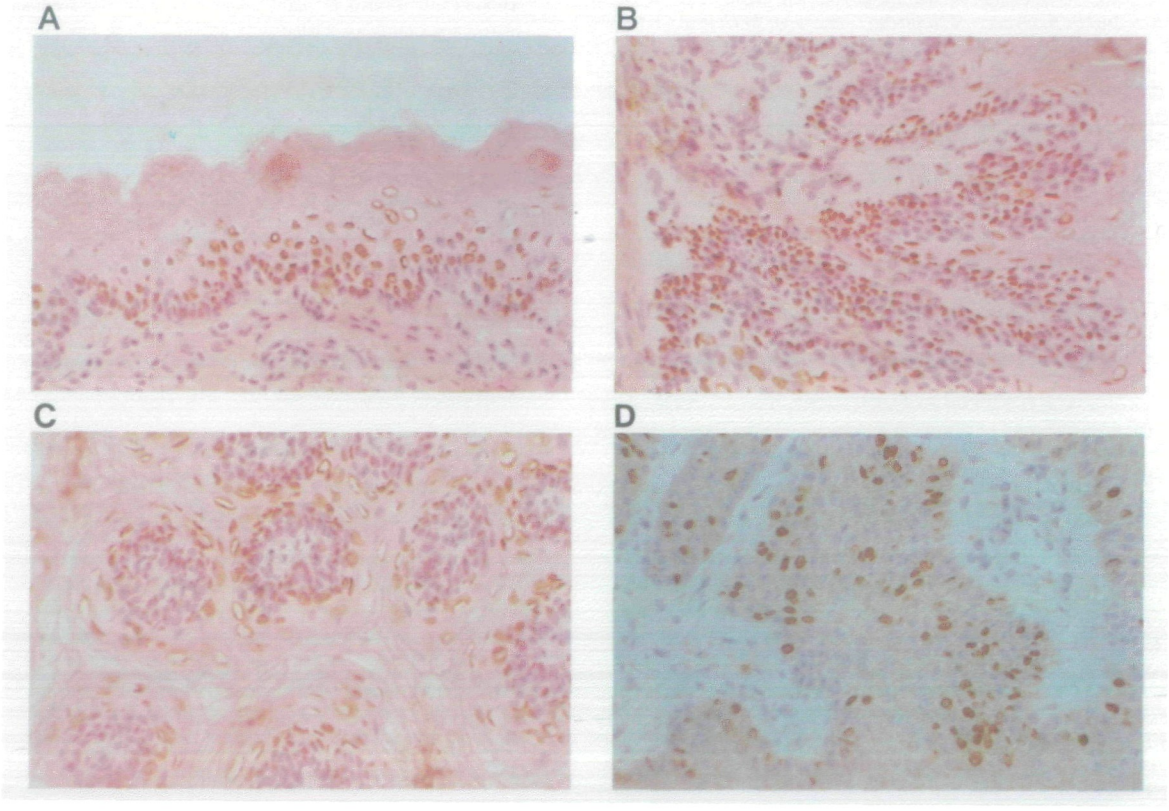

Fig. 2. *Immunocytochemical localization of O^6-methyldeoxyguanosine in hyperkeratotic regions of the oesophageal mucosa (A) and in oesophageal papillomas (B,C), induced by chronic exposure to NMBzA (10 p.p.m. in the drinking water), 6 h after a chasing dose of NMBzA (15 mg/kg body wt, i.p.). Localization of proliferating cells (D) with a single dose of BUdR (20 mg/kg body wt; survival time, 2 h) using a monoclonal antibody to BUdR. Counterstained with H&E (A–C) or haematoxylin (D). ×250.*

induced papillomas typically contain a GC to AT transition at the second base in codon 12 of the H-*ras* gene (26). In mouse skin it has been shown that the transition from papillomas to carcinomas is most efficiently mediated by continuous administration of an initiating agent, whereas tumour promotors, although effective in inducing clonal expansion of initiated cells, were ineffective in inducing further progression of the neoplasm (27). Similarly, selective expansion of initiated acetylaminofluore-resistant liver cells in female rats was rarely accompanied by progression events. However, the subsequent application of the directly alkylating carcinogen ethylnitrosourea, which does not lead to the induction of tumors in this tissue when given as the sole carcinogen (1), led to the formation of numerous neoplastic nodules within foci of initiated cells (28). These findings underline the importance of the capacity of preneoplastic and benign precursor lesions to bioactivate non-direct-acting carcinogens during the process of neoplastic progression.

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